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Cellular Locations of Proteinases and Association with Vesicles in *Porphyromonas gingivalis*

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Abstract
We found that locations of arginine-specific gingipain (RGP) in the cellular fractions in the crude extract, envelope, vesicles, and culture supernatants were 48%, 16%, 17%, and 31%, respectively, and the corresponding values of lysine-specific gingipain (KGP) were 47%, 10%, 7%, and 36%, respectively. Although the molecular mass of RGP in the culture supernatant had been determined as 43 kDa, and that of KGP had been as 48 kDa, molecular masses of both proteinases solubilized from the vesicles were estimated to be over 1,500 kDa, since they eluted in the void volume of the column in the gel filtration on Sephacryl S-300. There was no reduction of molecular size by the following treatment with SDS, high-concentration NaCl, or urea. Interestingly, the occurrence of the macromolecular forms could not observed in other enzymes tested such as monopeptidyl, dipeptidyl, and tripeptidyl peptidases, as well as alkaline phosphatase. Therefore, occurrence of the macromolecular forms may be restricted to the proteinases. When the vesicle and culture supernatants containing free RGP and KGP were mixed and incubated, neither RGP nor KGP seemed to bind to vesicles. RGP bound to the vesicle was found to be more stable to heat treatment than the free form, suggesting that association of RGP with the vesicle caused heat stability of this enzyme.

Key words: proteinase, enzyme, RGP, KGP, *P. gingivalis*, vesicle

Introduction

Gram-negative, black-pigmented obligatory anaerobes including *Porphyromonas gingivalis*, *Prevotella intermedia*, and *Prevotella nigrescens* have been implicated as etiological agents of human periodontitis, of which *P. gingivalis* is the most potent pathogen of this disease [1, 2, 3, 4]. RGP hydrolyses the peptide bonds of arginine-Xa.a. and KGP splits that of lysine-Ya.a., both are major proteinases of *P. gingivalis*, and these enzymes are considered to be important in the pathogenesis of periodontitis. The biochemical properties of these enzymes were described in the last decade and proteolytic enzymes have been implicated as important pathogenic factors [5, 6, 7]. However, observations from the biological aspects remain unsatisfactory.

Vesicles have been thought to originate from the outer membranes of gram-negative bacterial species. Toxic substances were found in the vesicles of *Aggregatibacter* (*Actinobacillus*) *actinomycetemcomitans* and they were considered to be released into the crevicular environment, which may contribute to the pathogenesis of this species [8, 9]. Grenier and Mayrand described the vesicles of *Porphyromonas gingivalis* in which they reported that vesicles of approximately 50 nm predominated and contained highly active enzymes against collagen, Azocoll, and a synthetic substrate of RGP. They also established methods of vesicle preparation from liquid cultures [10].

Vesicles of *P. gingivalis* from the outer membrane were prepared by a combination of mechanical disruption of the cells followed by 80,000 xg centrifugation and sarkosyl treatment of the cells, sonication, and 100,000 xg centrifugation. Extracellular vesicles were obtained by precipitation with ammonium sulfate from culture supernatant [11]. Sarkosyl-insoluble preparation and extracellular vesicles yielded similar protein patterns in SDS-PAGE. However, vesicles prepared directly from the cells contained additional proteins. The mechanism of formation and release of theses vesicles in *P. gingivalis* was proposed as a result of "herniation" of the outer membrane in the process of turnover of peptidoglycan [12].

We noticed that RGP and KGP are found in the culture supernatant, in the surfaces and within the cytoplasm. As for those in the culture supernatant, two types of enzymes exist: one is a free form and the other is bound to released vesicles. The free forms of RGP and KGP were isolated and their molecular masses were determined to be 43 kDa and 48 kDa, respectively [13,14]. Since vesicles of gram-negative bacteria may play an important role in the transportation of their virulence factors to the host cells [9], we attempted to undertake an investigation of the interaction of proteinases and the vesicles.

Materials and Methods

All procedures were conducted at 4 °C, if not otherwise specified.

Bacterial Strains and Cultivation

P. gingivalis ATCC 33277 was used mainly in this study although *P. gingivalis* W83 and 381 were also employed. These strains were maintained anaerobically at 37 °C on blood agar plates containing hemin (5 μg/ml) and menadione (0.5 μg/ml). The fresh cultures of the strains were inoculated into a medium containing Trypticase peptone (17 g/liter), yeast extract (3 g/liter), NaCl (5 g/liter), K_2HPO_4 (2.5 g/liter), hemin (5 mg/liter), and menadione (0.5mg/liter) and cultured at 37 °C in a glove box filled with a mixture of gases (N_2: H_2: CO_2; 85 : 10 : 5) for 2 days.

Preparation of Bacterial Fractions

Culture supernatant containing vesicles were prepared from the whole culture by centrifugation at 10,000 xg for 15 min. Vesicles were collected by the methods described by Grenier et al. [10] with minor modifications: ammonium sulfate was added to the culture supernatant at a concentration of 40% saturation of this reagent and stirred for 5 h. The mixtures were centrifuged at 25,000 xg for 20 min, and the precipitate was suspended in 50 mM Tris-HCl buffer, pH 8.2 and dialyzed against the same buffer. After dialysis, the suspension was centrifuged at 40,000 xg for 30 min and the precipitate was referred to as the vesicle fraction. The concentration of the supernatant of the 40% saturation of ammonium sulfate mixture was raised to 75% saturation; the precipitate was collected by centrifugation and dissolved in 50 mM Tris-HCl buffer, pH 8.2, followed by dialysis against the same buffer. This fraction was designated the concentrated culture supernatant. Analytical size of culture supernatant free from particles was prepared from the vesicle containing culture supernatant (10,000 xg supernatant of whole culture) by centrifugation at 100,000 xg for 60 min. On the other hand, cells were disrupted by sonication at 150 W for 20 min. Unbroken cells and debris in the sonicate were removed by centrifugation at 6,000 xg for 15 min, then the supernatant was centrifuged once more at 100,000 xg for 60 min. The resultant supernatant was designated crude extract and the precipitate was the envelope.

Solubilization of Vesicles and Envelope by Triton X-100

Triton X-100 was added to 0.5% to the suspensions of the vesicles and the envelope in 50 mM Tris-HCl buffer (pH 8.2) and stirred for 30 min at room temperature, followed by centrifugation at 100,000 xg for 1 h. The supernatant solutions were stored as solubilized fractions of vesicles and envelope.

Determination of Enzyme

Activities of arginine-specific proteinase (RGP) and lysine-specific proteinase (KGP) were estimated using chromogenic synthesized substrates, benzoylarginine-p-nitroanilide and tosyl-glycylprolyllysine-*p*-nitroanilide, respectively, accordingly our previous reports [13, 14]. Dipeptidyl peptidase activities were assayed using *p*-nitroanilide derivatives of glycyl-phenylalanine (DPP 1), lysyl-alanine (DPP 2), glycyl-proline (DPP 4). Proline specific tripeptidyl peptidase activity was determined with alanyl-alanyl-proline-*p*-nitroanilide. Alkaline phosphatase activity was measured photometrically using *p*-nitrophenylphosphate [15].

Purification of RGP of Culture Supernatant

Free form 43 kDa RGP and 48 kDa KGP of *P. gingivalis* ATCC 33277 in the culture supernatant were purified by the procedures including concentration by ammonium sulfate, ion-exchange chromatography, gel filtration, and isoelectric focusing [13, 14].

Partial Purification of Vesicle-Bound RGP and KGP

The solubilized materials of vesicle were applied to a column of Q-Sepharose (GE Healthcare UK Ltd) equilibrated with 50 mM Tris-HCl buffer, pH 8.5. After the column was washed with the same buffer, the proteins were eluted with a linear gradient of NaCl from 0 to 800 mM. The active fractions were concentrated and dialyzed against 50 mM Tris-HCl buffer, pH 8.0 containing 200 mM NaCl and subjected to gel filtration on Sephacryl S-300 HR (GE Healthcare) and eluted with this buffer saline. The eluates in the void volume of the column containing RGP and KGP activities were collected and designated partially purified RGP and KGP.

Gel Filtration

Recovered RGP and KGP activities from vesicle by Triton X-100 treatment were 72% and 68%, respectively. Sephacryl S-300 HR packed in a column (2.6 cm by 94 cm) was used for gel filtration of the solubilized materials. The column was eluted at a flow rate of 40 ml/h with 50 mM Tris-HCl buffer, pH 8.0 containing 200mM NaCl, if not otherwise stated. The samples to be subjected were dialyzed against this buffer saline before application to columns.

Treatment of Solubilized Materials of Vesicles by Several Reagents and Gel Filtration

Solubilized materials of vesicles were mixed separately with the following reagents, NaCl (0.5 M), SDS (1%), dithiothreitol (5 mM), and urea (6 M) in 50 mM Tris-HCl buffer, pH 8.0 and incubated at room temperature for 45 min. After the incubation, each mixture was applied to a column of Sephacryl S-300 HR (2.6 by 94 cm) which had been equilibrated with 50 mM Tris-HCl buffer, pH 8.0 containing each reagent and eluted with the same corresponding buffer.

Results

Locations of RGP and KGP

Yields of vesicle and envelope, and profiles of the cellular locations of RGP and KGP in culture supernatant, crude extract, vesicle, and envelope of three

Table 1. Cellular locations of RGP and KGP.

Porphyromonas gingivalis ATCC 33277 (cell: 12.4g)		RGP(U)	%	KGP(U)	%
culture supernatant*	2,000ml	155.5	47.6	78.2	47.0
crude extract	53ml	52.6	16.1	16.6	10.0
vesicle	1.00g	17.1	5.2	12.3	7.4
envelope	2.12g	101.8	31.1	59.1	35.6
total		327.0	100	166.2	100
Porphyromonas gingivalis W83 (cell: 11.3g)		RGP(U)	%	KGP(U)	%
culture supernatant*	2,000ml	72.0	37.7	50.0	39.9
crude extract	53ml	58.4	30.5	37.6	30.0
vesicle	0.86g	11.6	6.1	12.3	9.8
envelope	1.52g	49.2	25.7	25.5	20.3
total		191.2	100	125.4	100
Porphyromonas gingivalis 381 (cell: 12.9g)		RGP(U)	%	KGP(U)	%
culture supernatant*	2,000ml	126.0	59.0	88.0	58.7
crude extract	52ml	55.1	25.8	37.6	25.1
vesicle	0.72g	18.5	8.7	9.9	6.6
envelope	1.80g	13.9	6.5	14.4	9.6
total		213.5	100	149.9	100

*; particle free

strains of *P. gingivalis* are summarized in Table 1. Although the bacteria were found to grow as the same level, slight differences were observed in the yields of vesicle and envelope. RGP and KGP were detected in all fractions, and production of RGP appeared to exceed that of KGP in the three strains. The fraction containing the maximum amounts of these proteinases was the culture supernatants in all the three strains. Distribution of RGP and KGP in the four fractions were quantitatively similar, except the lower amounts of both proteinases in the envelope of strain 381.

Gel Filtration Profiles of Proteinases and Other Enzymes

The purified RGP of culture supernatant (43 kDa) eluted at elution volume of 335 ml in the gel filtration on Sephacryl S-300 (Fig. 1); however, the RGP solubilized from vesicles eluted in the void volume of the column (205 ml) with a negligible peak of the activity at the corresponding position of the purified culture supernatant RGP (Fig. 2). These findings indicate that the molecular mass of RGP originated from the vesi-

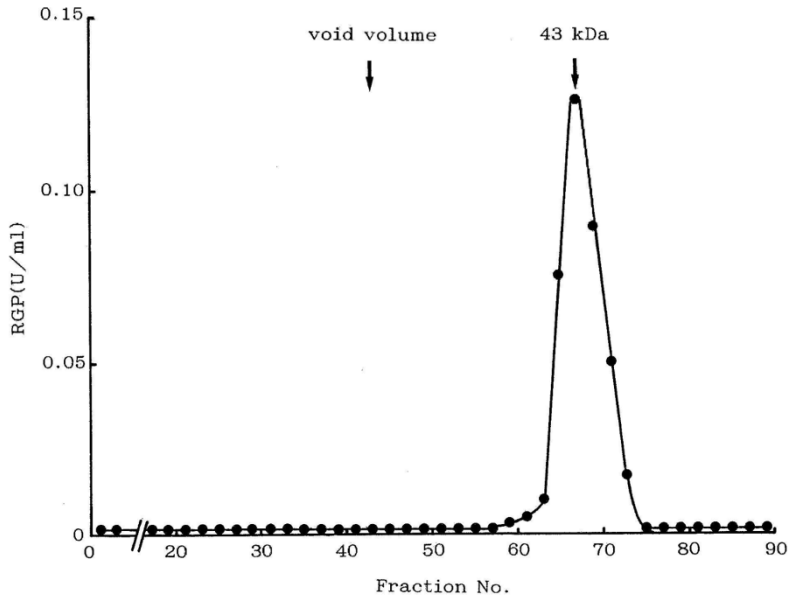

Fig. 1. Gel filtration on Sephacryl S-300 of RGP purified from culture supernatant. The column (2.6 by 94 cm) was eluted with 50 mM Tris-HCl, pH 8.0 containing 200 mM NaCl.

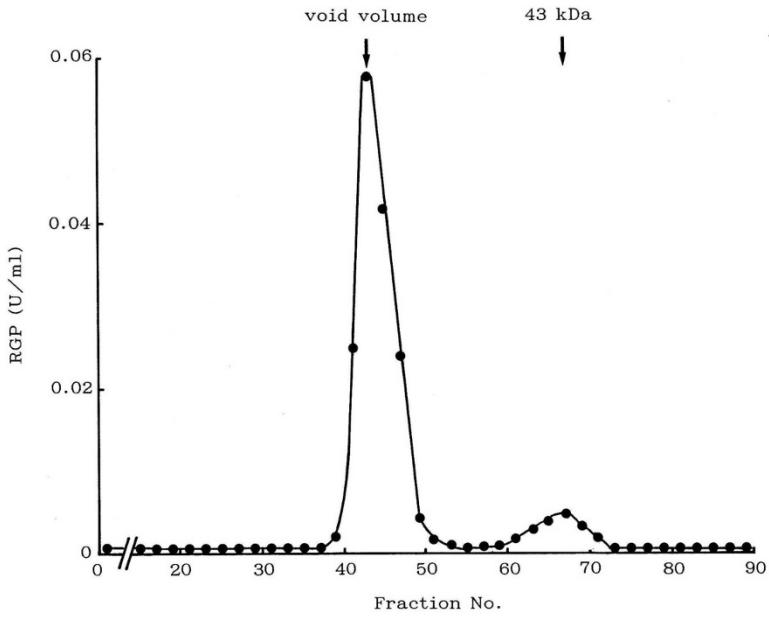

Fig. 2. Gel filtration on Sephacryl S-300 of RGP partially purified from the vesicle. The column (2.6 by 94 cm) was eluted with 50 mM Tris-HCl, pH 8.0 containing 200 mM NaCl.

Table 2. Elution positions of RGP, KGP, and some other enzymes in the gel filtration on Sephacryl S-300.

	void volume	valid volume
RGP		
crude extract	+	+
culture supernatant	-	+
vesicle extract	+	tr
envelope extract	+	+
KGP		
crude extract	+	+
culture supernatant	-	+
vesicle extract	+	+
envelope extract	+	+
Glycyl-phenylalaninyl-pNA (DPP 1)		
crude extract	-	+
culture supernatant	-	+
vesicle extract	-	+
envelope extract	-	+
Lysyl-alanyl-pNA (DPP 2)		
crude extract	-	tr
culture supernatant	-	-
vesicle extract	-	-
envelope extract	-	-
Glycyl-prolyl-pNA (DPP 4)		
crude extract	-	+
culture supernatant	-	-
vesicle extract	-	-
envelope extract	-	tr
Alanyl-alanyl-prolyl-pNA		
crude extract	-	+
culture supernatant	-	-
vesicle extract	-	tr
envelope extract	-	tr
Alkaline phosphatase		
crude extract	-	+
culture supernatant	-	+
vesicle extract	-	+
envelope extract	-	+

tr; trace

cles may be over 1,500 kDa (by the manual of Sephacryl S series). RGP eluted at the void volume was temporarily referred to as macromolecular form RGP. Gel filtration profiles of KGP were substantially the same as those of RGP; however, significant amounts of activity in vesicle sample were detected in the valid volume, and the approximate ratio of the activities in the void volume and to the valid volume was 2 to 1. To examine whether 43 kDa RGP was able to convert to the macromolecular form simply by incubation with vesicles, mixtures of vesicle and the purified 43 kDa or raw RGP (concentrated culture supernatant) were subjected to gel filtration, but no shift of the elution volume of 43 kDa RGP to the void volume was seen.

Efforts were made to assess the gel filtration profiles of other enzymes. As shown in Table 2, no activities were detected in the void volume position in the tested enzymes such as dipeptidyl peptidases (DPP-1, DPP-2, and DPP-4), tripeptidyl peptidase, and alkaline phosphatase. Theses were found in single peaks of the activities at the individual specified positions in the valid volume of the column, but not in the void volume.

Treatment of RGP solubilized from vesicles with NaCl (0.5 M), SDS (1%), dithiothreitol (5 mM), and urea (6 M) caused no effect on elution profiles of gel filtration. Activities in all cases were detected in the void volume, but not in the valid volume of the column of Sephacryl S-300 HR.

Comparisons of Heat Stability and Sensitivity to Enzyme Inhibitors of Free and Vesicle-Bound RGP and KGP

When the purified 43 kDa RGP was heated at 60 °C for 10 min, the residual activity was only 8.5% of the control unheated sample; however, the extracted RGP solubilized from the vesicle by detergent maintained 43.5% of the control activity. Whereas, both samples of KGP solubilized from vesicle and purified from

culture supernatant were completely inactivated by this heat treatment. No different sensitivity to the RGP inhibitors including EDTA, tosyl-L-lysine-chloromethyl ketone, antipain, leupeptin, *p*-hydroxy-mercuribenzoate, and L-*trans*-Epoxy-succinylleucyl-amido-(4-guanidino)butane was observed between the two types of RGPs. Difference of activation rate by glycyl-glycine was also not noticed.

Discussion

Proteolytic enzymes are considered to be important etiological factors in periodontitis; however, transportation mechanisms of the bacterial virulent substances to the host mucosa should also be noticed. Since vesicles are considered to play a vehicular role [8, 9], we tried to evaluate the presence of RGP and KGP in vesicles, as well as in other subcellular fractions and the surrounding culture medium. We confirmed that vesicles contained RGP and KGP, even if both enzymes accounted for only small rates in the subcellular fractions (Tab1e 1).

Fujimura et al. reported that in the early stage of cultivation of *Staphylococcus aureus*, the molecular mass of staphylokinase in the culture supernatant was determined by gel filtration as 15 kDa (Type A); however, in the late stage, it appeared to convert to 320 kDa (Type B), indicating that Type B staphylokinase might be a complex of some high-molecular size substance. This complex could be dissociated by treatment with high concentrations of KCl or detergent [16]. However, formation of macromolecular mass RGP described in this report could be caused in a different manner from the conversion of molecular size of staphylokinase. We confirmed that RGP solubilized from the vesicles taken in the early stage of cultivation (15 h) also was expelled from the molecular sieving on Sephacryl S-300 (data not shown).

No difference in effect of inhibitors and activators on the free form and vesicle-bound form of RGP was observed, but a significant difference of thermostability was seen in these two forms of RGP. In other words, vesicles contributed to the thermostability of RGP. The reason why the vesicle-bound RGP was more stable in the heat treatment than free RGP remains unclear. It is also unexplained why the effect of vesicle on the thermostability was not observed in KGP. However, it may be related to the observations that envelope-associated RGPs were found to be more stable than KGP (17).

Recently, *P. gingivalis* outer membrane vesicles were observed entering HeLa cells and surviving within the endocytic organelles (18). We attempted also to evaluate the binding of vesicles to mammalian cells using human epithelial cell A549, cultured in Dullbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) under 5% CO_2 for 7 days and red blood cell of horse, sheep, rabbit, and human. Binding was assessed by reduction of RGP activity associated to the vesicles in the centrifugal supernatants at 1,000 xg of the reaction mixtures of cell suspensions and vesicles, based on the previous observation that these cells are precipitated by low speed centrifugation, but that vesicles remained in the supernatant. However, we could confirm significant binding of the vesicles neither to epithelial cells nor to red blood cells.

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